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# Evidence for Involvement of Tumor Necrosis Factor-α in Apoptotic Death of Bone Marrow Cells in Myelodysplastic Syndromes

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**We previously reported excessive apoptosis and high levels of tumor necrosis factor-alpha (TNF-α) in the bone marrows of patients with myelodysplastic syndromes (MDS), using histochemical techniques. The present studies provide further circumstantial evidence for the involvement of TNF-α in apoptotic death of the marrow cells in MDS. Using our newly developed in situ double-labeling technique that sequentially employs DNA polymerase (DNA Pol) followed by terminal deoxynucleotidyl transferase (TdT) to label cells undergoing apoptosis, we have characterized DNA fragmentation patterns during spontaneous apoptosis in MDS bone marrow and in HL60 cells treated with TNF-α or etoposide (VP16). Clear DNA laddering detected by gel electrophoresis in MDS samples confirmed the unique length of apoptotic DNA fragments (180–200 bp). Surprisingly, however, phenotypically heterogeneous population of MDS cells as well as the homogenous population of HL60 cells showed three distinct labeling patterns after double labeling—only DNA-Pol reaction, only TdT reaction, and a combined DNA Pol + TdT reaction, albeit in different cohorts of cells. Each labeling pattern was found at all morphological stages of apoptosis. MDS mononuclear cells, during spontaneous apoptosis in 4 hr cultures, showed highest increase in double-labeled cells (DNA Pol + TdT reaction). Interestingly, this was paralleled by TNF-α–induced apoptosis in HL60 cells. In contrast, VP16 treatment of HL60 cells led to increased apoptosis in cells showing only TdT reaction. The double-labeling technique was applied to normal bone marrow and peripheral blood mononuclear cells after treatment with known endonucleases that specifically cause 3′ recessed (*Bam*HI), 5′ recessed (*Pst*I), or blunt ended (*Dra*I) double-stranded DNA breaks. It was found that the DNA-Pol reaction in MDS and HL60 cells corresponds to 3′ recessed DNA fragments, the TdT reaction to 5′ recessed and/or blunt ended fragments, and a combined ''DNA Pol + TdT reaction'' corresponds to a copresence of 3′ recessed with 5′ recessed and/or blunt ended fragments. Clearly, therefore, apoptotic DNA fragments, in spite of a unique length, may have differently staggered ends that could be cell (or tissue) specific and be selectively triggered by different inducers of apoptosis. The presence of TNF-α–inducible apoptotic DNA fragmentation pattern in MDS supports its involvement in these disorders and suggests that anti–TNF-α (or anticytokine) therapy may be of special benefit to MDS patients, where no definitive treatment is yet available. Am. J. Hematol. 60:36–47, 1999.** 

**Key words: human disorders; myelodysplastic syndromes (MDS); HL60cells; apoptosis; DNA fragmentation; in situ labeling; tumor necrosis factor-α (TNF-α); etoposide (VP16); hematopoietic disorders**

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## INTRODUCTION

In the past we showed that hematopoietic as well as stromal cells in the bone marrows (BMs) of patients with myelodysplastic syndromes (MDS) demonstrate excessive apoptotic death, thus forming the basis for ineffective hematopoiesis observed in these disorders [1–3]. Others have confirmed our observation of high intramedullary apoptosis in MDS [4,5]. Such a widespread incidence of apoptosis affecting all types of bone marrow cells was indicative of a possible involvement of cytokine(s) with a broad range of target cells. Using immunohistochemistry, we indeed detected high levels of tumor necrosis factor-alpha (TNF-α) correlating significantly with the levels of apoptosis detected in situ in the same BM biopsies of MDS patients [3]. In our attempts to further understand the association between TNF-α and apoptosis in MDS, in the present studies we examined the patterns of DNA fragmentation during spontaneous apoptosis in MDS and in TNF-α–induced apoptosis in HL60 cells.

Internucleosomal DNA fragmentation constitutes one of the most salient features of apoptotic cell death. The four endonucleases most prominently implicated in apoptotic DNA fragmentation are $Ca^{++}$ $Mg^{++}$ dependent, $Mg^{++}$ dependent, $Mn^{++}$ dependent, and acidic endonuclease. These endonucleases have been shown to maintain the property of internucleosomal cleavage [6–8]. No specific differences with respect to substrate requirement or products formed have been noted among different apoptotic endonucleases. The DNA fragments formed by such endonucleolytic activity that typically appear to have 5′-P and 3′-OH terminals [9–11], could be detected using two specific enzymatic reactions that label the ends of DNA fragments. One of these techniques uses DNA Polymerase (DNA Pol) or Klenow fragment of DNA Pol [10] whereas the other uses terminal deoxynucleotidyl transferase (TdT) for this purpose [9]. We have extensively used these enzymatic reactions to detect apoptosis in situ in a variety of clinical samples [1–3,12,13]. In one of these studies, we compared the labeling of apoptosis by the two enzymatic reactions performed separately in serial sections of different types of solid tumors. It was found that some tissues like breast tumors or primary brain tumors did not show labeling with DNA Pol, but showed positivity with TdT labeling [12,13]. On the other hand, tissues like nonHodgkin's lymphoma or head and neck squamous cell carcinoma showed comparable labeling by the two enzymes in serial biopsy sections [12]. Gold et al. [14] in 1994 reported that whereas DNA Pol preferentially labels necrosis, TdT was more specific for apoptosis. Contrastingly, we found that both methods label apoptosis as well as necrosis except that necrosis is labeled with an extremely low intensity by both methods [12,15].

To explain the tissue-specific differences in labeling with DNA Pol and TdT noted in our studies, we hypothesized that the apoptotic DNA fragments may have a unique length of integral multiples of 180–200 bp, but have differently staggered ends—3′ recessed, 5′ recessed, or blunt—perhaps reflecting the specificity of the endogenous endonuclease(s) involved. To test this postulate we developed an in situ double-labeling technique in which the DNA-Pol reaction is performed first, followed by the TdT reaction. Using this technique, as described in the present paper, we have now been able to confirm our hypothesis and characterize and compare the ends of apoptotic DNA fragments in spontaneous apoptosis in MDS with that induced by TNF-α in HL60 cells. The findings of these studies reported in the present paper highlighting their therapeutic implications suggest a novel approach to the treatment of MDS.

## MATERIALS AND METHODS

### Patients

Sixteen bone marrow aspirate specimens from 15 patients with a confirmed diagnosis of MDS [diagnosed according to the French-American-British classification as refractory anemia (RA)-7, RA with ringed sideroblasts (RARS)-3, RA with excess of blasts (RAEB)-3, RA with excess of blasts in transformation (RAEBt)-1, and Chronic myelomonocytic leukemia (CMMoL)-1] were studied for the incidence of spontaneous apoptosis. One MDS patient (RARS) was studied on two occasions. Six normal bone marrows from healthy donors were studied for comparison. The protocols, MDS 90 02 and MDS 95 01, under which clinical specimens were obtained, were approved by the local Institutional Review Board (IRB) and informed consent was obtained from the donors. Bone marrow aspirates were subjected to Ficoll-Hypaque density gradient centrifugation to separate mononuclear cells. After confirming the viability by trypan blue dye exclusion test, cells were suspended ($1 \times 10^6$ cells/ml) in RPMI-1640 medium (GIBCO-BRL Life Technologies Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM/l glutamine (complete medium). One aliquot was used for 0 hr tests and the other was incubated for 4 hr at 37°C in the presence of 5% $CO_2$. At both time points, cells were washed and divided into two aliquots. One was fixed on alcian blue coated coverslips using 4% buffered paraformaldehyde (pH 7.1) overnight at 4°C and stored in 70% ethanol until in situ tests for detection of apoptosis were performed. The other aliquot was used to extract DNA following cell lysis in guanidine isothiocyanate (GITC) as described previously [12].

## HL60 Cell Cultures

HL60 cells were maintained in RPMI-1640 complete medium supplemented with 20% FBS. Freshly harvested cells, after confirming viability by trypan blue dye exclusion test, were resuspended ($1 \times 10^6$ cells/ml) in complete medium and treated with human recombinant TNF-α (0.01 ng/ml; Promega Inc., Madison, WI) for 8 hr or with VP16 (etoposide—35 μM/l; Bristol Laboratories, Princeton, NJ) for 4 hr. As both these agents were dissolved in RPMI 1640, cells suspended in plain complete medium and incubated for a maximum interval, i.e., 8 hr served as controls. The dose of TNF-α was chosen based on the $ED_{50}$ dose (0.016 ng/ml) suggested by the manufacturer and according to our initial dose response experiments, which showed a linear apoptotic response at 0.01 ng/ml until 8 hr (data not shown). The concentration and incubation time of VP16 was based on our previous studies [12]. Cells were fixed on alcian blue coated coverslips using 4% paraformaldehyde before and after the designated time of incubation, and stored in 70% ethanol for in situ detection of apoptosis. Experiments were repeated four times and the results represent the average of four experiments.

## Single Labeling of Apoptosis With DNA Pol I or TdT

These techniques to detect apoptotic cell death in situ have been described in detail in previous reports by us [1–3,12] and others [9,10]. Briefly, cells were rehydrated, postfixed in 0.23% periodic acid, and pretreated with SSC solution (30 mM/l sodium citrate + 0.3 M/l sodium chloride, pH 7.0) at 80°C for 20 min. The cells were then treated with either a mixture of deoxyribonucleotides, one of which was biotinylated (11 bio-dUTP; Sigma, St. Louis, MO) and E. coli DNA Pol I (Promega, Madison, WI) or with 11 bio-dUTP and TdT (Promega). Incorporation of labeled nucleotide was visualized using avidin-biotin-horseradish peroxidase (Vectastain Elite ABC kit, Vector, Burlingame, CA) and diaminobenzidine tetrahydrochloride (DAB). Dark brown nuclear staining indicated cells that were undergoing apoptotic death.

## Double Labeling With DNA Pol I and TdT

Cells were rehydrated, postfixed with 0.23% periodic acid, and pretreated with SSC solution. Subsequently, cells were washed in buffer A, pH 7.5 (50 mM/l Tris hydrochloride, 5 mM/l magnesium chloride, 10 mM/l β mercaptoethanol, and 0.005% bovine serum albumin, fraction V) (Sigma) and treated with a cocktail of four deoxynucleotides, one of which was biotinylated (bio dUTP) and DNA Pol I prepared in buffer A [0.01 mM/l of dATP, dCTP, and dGTP (Promega) + 0.001 mM/l bio dUTP (Sigma) and 20 U/ml *Escherichia coli* DNA Pol I (Promega)] at 18–19°C for 2 hr. The DNA-Pol reaction was visualized with ABC and DAB giving brown nuclear staining. Subsequently, cells were washed with buffer B, pH 6.8 (100 mM/l sodium cacodylate, 0.1 mM/l dithiothreitol, 5 mM/l cobalt chloride) (Sigma) and treated with a mixture of TdT (10 U/ml) and 0.001 mM/l digoxigenin-dUTP (Boehringer Mannheim, Germany) prepared in TdT buffer. The reaction was performed at 37°C for 1 hr and was terminated by immersing the coverslips in SSC solution. Incorporation of digoxigenin-dUTP was detected using antidigoxigenin Fab fragment conjugated with alkaline phosphatase (1:100 diluted; Boehringer Mannheim, Germany). The cells were then immersed in a solution freshly prepared as follows: Naphthol As-Mx Phosphate (20 mg; Sigma) was dissolved in 2 ml of N-N′-dimethylformamide (Sigma) and this was added to 100 ml of 0.1 M/l Tris buffer, pH 8.2, at 20°C. Next, 0.1 ml of 1 M/l levamisole was added to the solution to inhibit endogenous alkaline phosphatase, followed by 100 mg of Fast blue BB salt (Sigma). This mixture was stirred for 2 min and then filtered. The coverslips were then immersed in this solution for 10–12 min. The coverslips were finally washed in distilled water and mounted in fluoromount. Positive TdT reaction thus stained nuclei blue.

## Controls for In Situ Labeling Procedures

Specimens treated with the in situ end labeling reaction mixtures prepared in respective buffers with labeled nucleotide (and nucleotides for DNA Pol), but devoid of the enzyme (DNA Pol or TdT), served as negative assay controls.

**Treatment of normal human bone marrow and peripheral blood mononuclear cells with known endonucleases.** Normal bone marrow was collected from a resected rib procured during a thoracic surgical procedure and peripheral blood was obtained from a healthy donor under IRB approved protocols and with donors' written consent. Mononuclear cells were separated by density centrifugation and fixed on alcian blue coated coverslips with 4% paraformaldehyde and stored in 70% ethanol. The cells were rehydrated, postfixed with 0.23% periodic acid and pretreated with SSC solution as described above. At this point cells were subjected to differential treatments with known sequence specific endonucleases (Boehringer Mannheim) in respective reaction buffers provided along with the enzymes and at the overdigestion concentrations recommended by the manufacturer as follows:

1. *Bam*HI (causes 3′ recessed breaks with 5′ overhangs—5′-G↓GATCC-3′) at 40 U/100 μl for 16 hr at 37°C.
2. *Pst*I (causes 5′ recessed breaks with 3′ overhangs—5′-CTGCA↓G-3′) at 120 U/100 μl for 16 hr at 37°C.

3. *Dra*I (causes blunt ended breaks—5′-TTT↓AAA-3′) at 120 U/100 μl for 16 hr at 37°C.
4. *Pst*I (120 U/100 μl) for 16 hr at 37°C followed by *Bam*HI (20 U/100 μl) for 2 hr at 37°C.
5. Mixture of *Pst*I + *Dra*I (120 U/100 μl each) for 16 hr at 37°C.

At the end of treatments, cells were washed thoroughly with phosphate buffered saline and continued with double labeling as described above. Experiments were repeated to confirm the results.

**Determination of labeling index.** Every specimen was carefully observed under light microscopy and 1–2 thousand cells from several randomly selected 100× objective fields were counted to determine the percent of labeled cells in each case. The significance of differences in the mean percent labeling indices in various test groups was determined by the Student's *t* test. Also, the paired *t* test was used to determine the significance of differences in relative percent increase in labeling index of individual labeling patterns within each study group.

## RESULTS

### Spontaneous Apoptosis in MDS

Our earlier studies showed an excessive incidence of spontaneous apoptosis in the bone marrows of MDS patients in which a significant number of mononuclear cells from the bone marrow aspirates of these patients underwent apoptosis in 4-hr cultures in complete medium containing 10% serum [1,2]. Therefore, in the present study comparative labeling of Ficoll-separated mononuclear cells from 16 MDS aspirates and six normal aspirates, with DNA Pol and TdT was examined at 0 and 4 hr following incubation in vitro in a complete medium. Staining was performed after paraformaldehyde fixation at each time point. As shown in Figure 1, the mean labeling index of MDS cells at 4 hr by either enzymes was significantly higher than that at 0 hr. Furthermore, the labeling by the two enzymes at each time point was comparable (0.8% ± 0.4% at 0 hr vs. 3.9% ± 0.9% at 4 hr, n = 16, $P = 0.004$, by DNA Pol, and 1.3% ± 0.4% at 0 hr vs. 3.8% ± 1.0% at 4 hr, n = 10, $P = 0.046$, by TdT). Interestingly, normal cells also showed a marginal but significant increase in labeling index in 4 hr (0.1% ± 0.07% at 0 hr vs. 0.8% ± 0.1% at 4 hr, n = 6, $P = 0.001$, by DNA Pol, and 0.2% ± 0.08% at 0 hr vs. 1.5% ± 0.2% at 4 hr, n = 5, $P = 0.005$, by TdT). It is evident that at 4 hr, the labeling indices of MDS cells were 2–3 times higher than those of normal cells ($P = 0.003$ for DNA Pol and $P = 0.05$ for TdT), hence confirming the increased propensity of MDS bone marrow cells to undergo spontaneous apoptosis in vitro.

We subsequently performed our newly developed enzymatic double labeling with DNA Pol/diaminobenzidine (brown staining) and TdT/fast blue (blue staining) systems, at the two time points on nine MDS and five normal specimens. Three distinct labeling patterns were observed both in MDS and normal specimens—cells with nuclei stained only brown (only DNA-Pol reaction), only blue (only TdT reaction), and double labeled (DNA Pol + TdT reaction). Interestingly, as depicted in Figure 2, each labeling pattern was found at all phases of apoptosis ranging from the early-stage nuclear margination to intermediate-stage chromatin condensation and clumping, to the end-stage karyorrhexis, indicating the maintenance of labeling pattern throughout the process of apoptosis at a single cell level. The increase in mean labeling index in 4 hr, for each individual pattern was significant in MDS (Fig. 3a). In contrast, normal cells did not show a significant increase in individual patterns, but the increase in total index reached statistical significance (Fig. 3b).

Parallel to these experiments, DNA fragmentation was also studied by agarose gel electrophoresis (MDS, n = 8 and normal, n = 2). As illustrated in Figure 4, at 0 hr neither the MDS specimen (lane 2) nor the normal cells (lane 5) showed low molecular weight DNA fragments. However, after 4 hr MDS cells showed an intensely

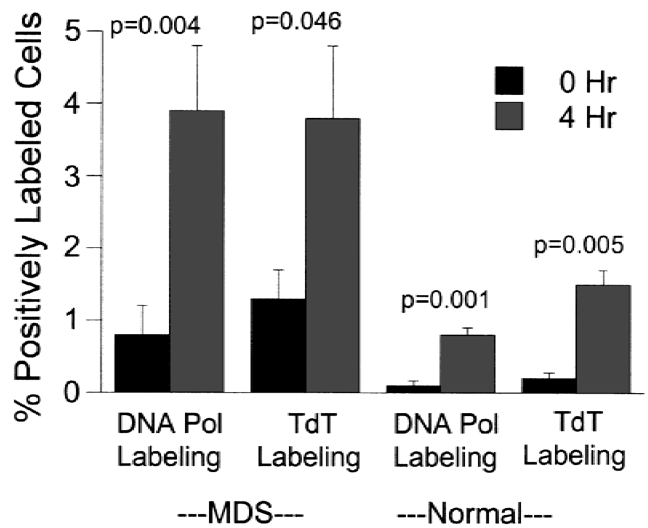


**Fig. 1. Comparative detection of apoptosis in MDS and normal cells using DNA Pol or TdT single labeling: Bone marrow aspirate mononuclear cells from MDS and normal subjects were incubated in RPMI 1640 containing 10% FBS for 4 hr. Spontaneous apoptotic death was detected in these cells following fixation in 4% buffered paraformaldehyde, using single labeling with DNA Pol or TdT and bio-dUTP. Percent positively labeled cells (dark brown staining in the nuclei) were determined in each case. By either technique, the labeling index was significantly higher at 4 hr in MDS as well as in normal, but those in the former were 2–3 times higher than the latter, indicating higher propensity of MDS cells to undergo spontaneous apoptosis. No significant difference was noted in the labeling efficiencies of the two enzymes at either time points within each group.**

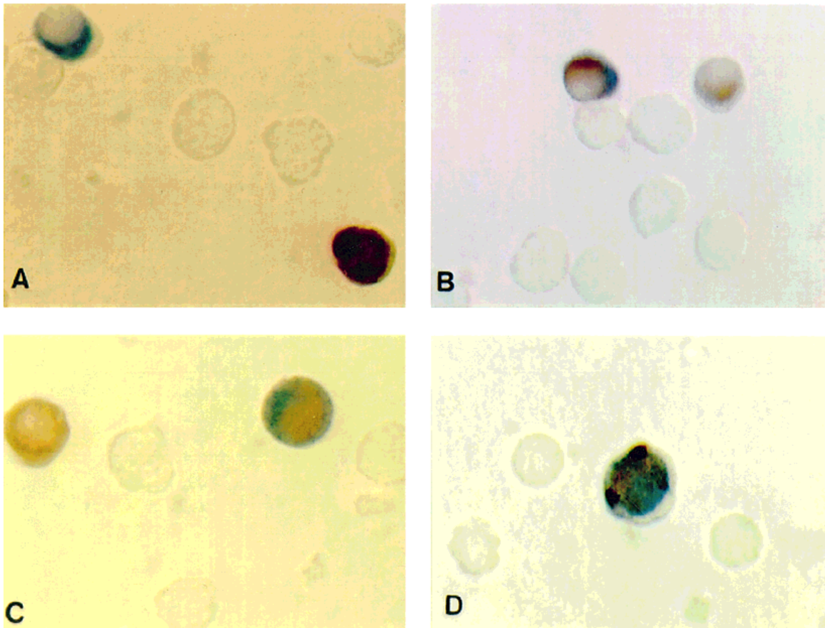


**Fig. 2. Double labeling of bone marrow aspirate mononuclear cells with DNA Pol/biotin-dUTP/diaminobenzidine (brown staining) and TdT/digoxigenin-dUTP/Fast blue (blue staining): As depicted here, both in MDS and normal specimens, three distinct labeling patterns were recognized: only-brown, only-blue, and double labeled. Furthermore, interestingly enough, each pattern was seen at early as well as late stages of apoptosis ranging from nuclear margination at early stage to larger clumping and fragmentation at late stages, e.g., double-labeled cells at early (B), intermediate (C), and late (D) stages or brown labeling at early (C) and late (A) stages seen in these micrographs. Original magnification, ×1000.**

stained, characteristic ladder of low molecular weight DNA fragments (lane 3), whereas the two normal specimens studied showed only a faint ladder (lane 6). One of the MDS specimens studied showed laddering at 0 hr also. The presence of clear laddering and lack of smearing in each case shows the absence of necrosis and confirms apoptosis.

### TNF-α Vs. VP16-Induced Apoptosis in HL60 cells

DNA fragmentation patterns were also studied in HL60 cells induced to undergo apoptosis by treatment with TNF-α (0.01 ng/ml for 8 hr) or VP16 (0.35 μM/l for 4 hr). Cells incubated with vehicle only (culture medium) for the highest incubation period of 8 hr, served as controls. The experiments were repeated four times. Controls however were available from three experiments. DNA Pol/TdT double labeling was performed after paraformaldehyde fixation of cells before and after treatment in each experiment. The results described here represent the average of four experiments. Surprisingly, like MDS or normal cells, even the HL60 promyelocytic cells showed three distinct labeling patterns in each group. In the untreated group, during the 8-hr incubation, there was a slight increase in cells with only-DNA Pol reaction, a notable increase in cells with only-TdT reaction, and virtually no increase in double-labeled cells (Fig. 5a). On the other hand, TNF-α treatment induced an appreciable increase in the number of cells with only-DNA Pol reaction, virtually no increase in cells with only-TdT reaction, and a significant increase in double-labeled cells (DNA Pol + TdT reaction) and in total labeling index (Fig. 5b). In contrast, VP16 treatment showed the highest labeling indices with a remarkable increase in cells with only-TdT reaction and only a marginal increase in the other two patterns (Fig. 5c), with a significant increase in the total labeling index. Thus, at the end of the designated period of incubation, among individual labeling patterns, untreated cells and VP16-treated cells showed the highest labeling indices in cells with only-TdT reaction, whereas TNF-α treated cells showed the highest indices in double-labeled cells (DNA Pol + TdT reaction).

### Comparison of Relative Percent Increase in Different Labeling Patterns in MDS, Normal, and HL60 Cells

Considering the total net increase in percent positively labeled cells as 100%, relative percent increase in individual pattern was calculated in each case. Figure 6 compares the relative percent increase in individual labeling patterns during spontaneous apoptosis of MDS and normal cells, and in HL60 cells with or without treatment. Normal cells showed a comparable increase in the three labeling patterns (only-DNA Pol reaction—30.6% ± 19.5%; only-TdT reaction—39.8% ± 18.8%, and double labeled (DNA Pol + TdT reaction)—29.6% ± 11.2%, n = 5). On the other hand, MDS cells showed twice as much increase in double-labeled cells and in cells with only-DNA Pol reaction as compared with the cells with only-TdT reaction (39.2% ± 6.8%, $P = 0.08$ and 38.6% ± 5.3%, $P = 0.06$ vs. 22.3% ± 6 % respectively, n = 9). Surprisingly, TNF-α–treated HL60 cells exhibited a similar pattern to spontaneous apoptosis in MDS, showing the highest increase in double-labeled cells and the

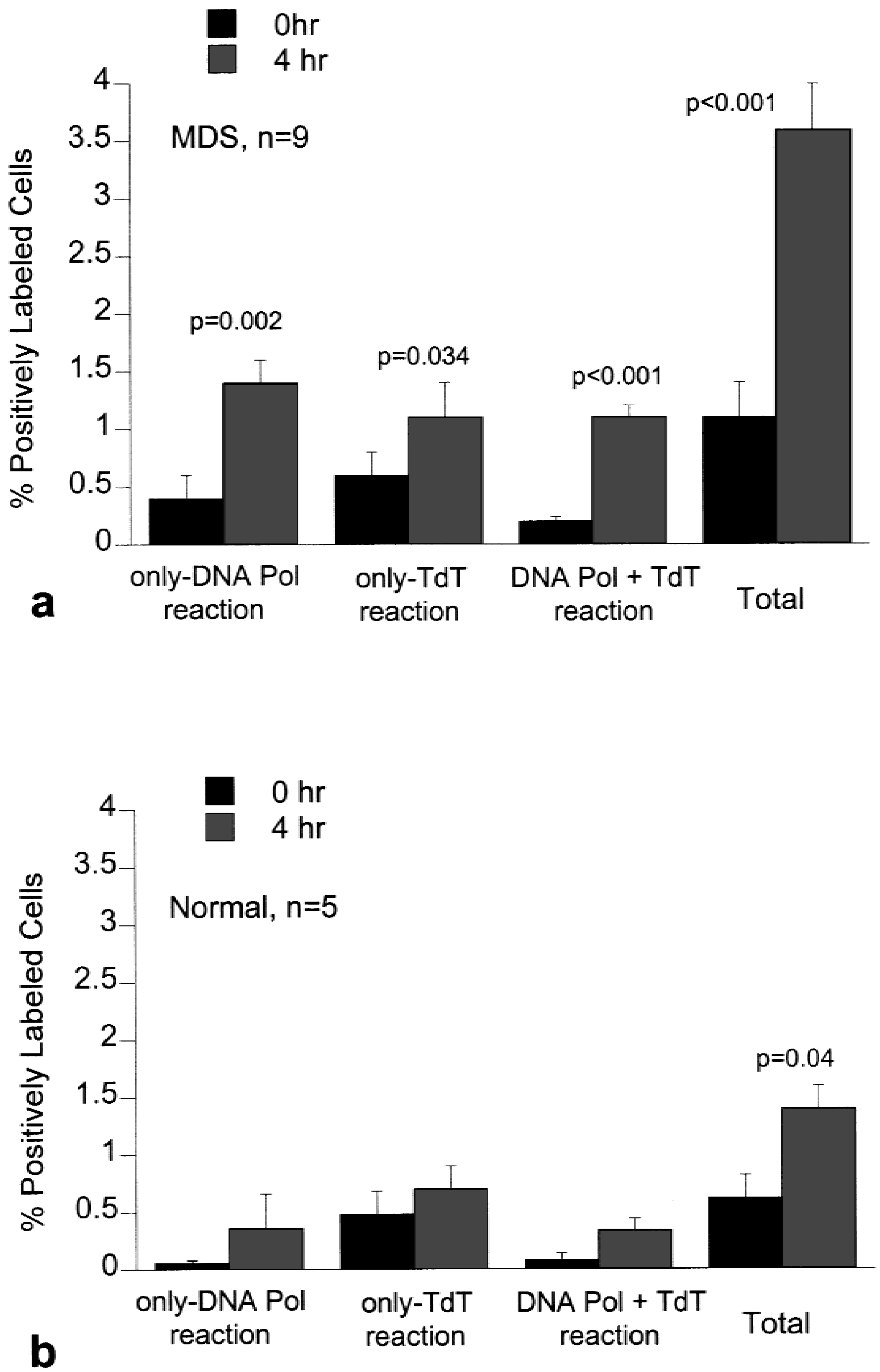


**Fig. 3. Increase in individual labeling patterns in MDS and normal cells: Percentage of cells labeled positively for each individual staining pattern were determined in MDS (a) and normal specimens (b). Note the significant increase observed in 4 hr in each individual labeling pattern in MDS cells as compared with the normal cells, which showed marginal increase only in the total labeling index.**

least increase in cells with only-TdT reaction (double labeled—47.3% ± 4.4%, $P = 0.015$ and only-DNA Pol reaction—32.2% ± 8.2%, $P = 0.3$ vs. only-TdT reaction—20.5% ± 6.6%, $n = 4$). The untreated and VP16-treated HL60 cells qualitatively showed a comparable pattern with relatively highest increase in cells with only-TdT reaction (69.9% ± 8.9%, $P \leqslant 0.01$, $n = 3$ and 62.6% ± 15.5%, $P \leqslant 0.07$, $n = 4$ respectively), albeit quantitatively, the labeling indices after VP16 treatment were 10 times higher than those of untreated cells.

## Determination of Specific DNA Breaks Labeled by Individual Staining Patterns

To determine if the individual staining patterns observed in our double-labeling experiments were related to specific types of DNA breaks, we treated normal human bone marrow—and peripheral blood—mononuclear cells with different known sequence-specific endonucleases (also see Methods section and the legend to Figure 7 for experimental details). Figure 7 shows staining in

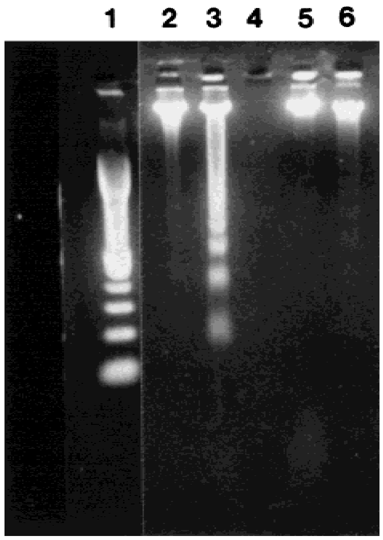


**Fig. 4. DNA ladder in MDS and normal cells: Agarose gel electrophoresis of DNA extracted from MDS and normal mononuclear cells, incubated for 4 hr in RPMI-1640 medium containing 10% FBS. (Ethidium bromide staining photographed in ultraviolet light). At 0 hr neither MDS (lane 2) nor normal cells (lane 5) showed the presence of low molecular weight DNA bands. At 4 hr, whereas MDS cells showed intensely stained low molecular weight bands (lane 3), the normal cells showed only a faint ladder (lane 6), suggesting the internucleosomal DNA cleavage causing fragments of unique length.**

normal bone marrow mononuclear cells under different conditions, which was paralleled by that in peripheral blood cells (data not shown). These bone marrow mononuclear cells virtually showed no labeling in the absence of nucleolytic treatment (Fig. 7). Upon treatment with the three endonucleases, however, these cells showed a specific staining pattern in each case, following in situ double labeling with DNA Pol and TdT. As illustrated in Figure 7, treatment with *Bam*HI, which gives rise to 3′ recessed double-stranded DNA fragments with 5′ overhangs, showed only-DNA Pol specific brown staining of the nuclei. On the other hand, treatment with *Pst*I that gives rise to double-stranded 5′ recessed DNA fragments with 3′ overhangs, or with *Dra*I which generates blunt ended double-stranded DNA fragments, exclusively showed only-TdT specific blue staining. Interestingly, when cells were treated with both *Pst*I and *Bam*HI, as shown in Figure 7, the majority of positive cells were double labeled (DNA Pol + TdT reaction), whereas treatment with *Pst*I and *Dra*I demonstrated only-blue labeling (only-TdT reaction). Surprisingly, in the former case, double labeling was seen only when cells were treated sequentially with *Pst*I first followed by *Bam*HI, while a concomitant treatment with the two enzymes showed dominance of *Bam*HI giving only-brown labeling (only-DNA Pol reaction; data not shown). On the other hand, sequential as well as concomitant treatment with *Pst*I and *Dra*I showed blue labeling only (only-TdT reaction). These experiments thus suggest that the brown labeling in MDS, normal, or HL60 cells could be due to the presence of 3′ recessed DNA fragments, whereas the blue labeling may be due to the presence of 5′ recessed and/or blunt-ended DNA fragments. Furthermore, the double labeling could be due to the copresence of 3′ recessed DNA fragments with 5′ recessed or blunt-ended DNA fragments.

## DISCUSSION

The mainstay in the therapy of MDS continues to be supportive care. Only through an in-depth understanding of the pathobiology of this intriguing disorder that we can hope to develop novel approaches in its treatment. Our present report not only provides a number of interesting insights into the biology of MDS and also into the process of apoptotic DNA fragmentation in general, but also shows a new ray of hope in the development of treatment for MDS. The most salient findings of these studies are summarized below and are subsequently discussed in detail:

1. The prominent DNA fragmentation pattern observed in spontaneous apoptosis of bone marrow cells in MDS is similar to that brought about by TNF-α treatment of HL60 cells.
2. Apoptotic DNA fragments may have a unique length but have differently staggered ends.
3. The two commonly used enzymes, DNA Pol and TdT, in a sequential double-labeling technique are now shown to differentiate the end patterns of apoptotic DNA fragments. DNA Pol can detect only 3′ recessed DNA fragments, whereas TdT applied subsequently, detects 5′ recessed and blunt-ended fragments. This observation defining the detection specificities of the two enzymes may warrant a caution in the current indiscriminate use of either enzyme singularly for detection of apoptosis.
4. The characteristic DNA fragmentation pattern at a single cell level may be conserved throughout the process of apoptotic DNA disintegration.
5. Different apoptotic stimuli could cause distinct DNA fragmentation patterns.

**Apototic death in the BMs of MDS patients demonstrates TNF-α–inducible DNA fragmentation pattern.** In the present study apoptosis was studied in density separated mononuclear cells from bone marrow aspirates of MDS patients. The extent of apoptosis determined in *bone marrow aspirate cells* during very short-term (only 4 hr) in vitro incubation conditions appears to be lower than the estimates reported previously in bone marrow biopsies by in situ studies by us and others [1,3,5]. However, our present estimates are in concordance with the estimates reported previously by us

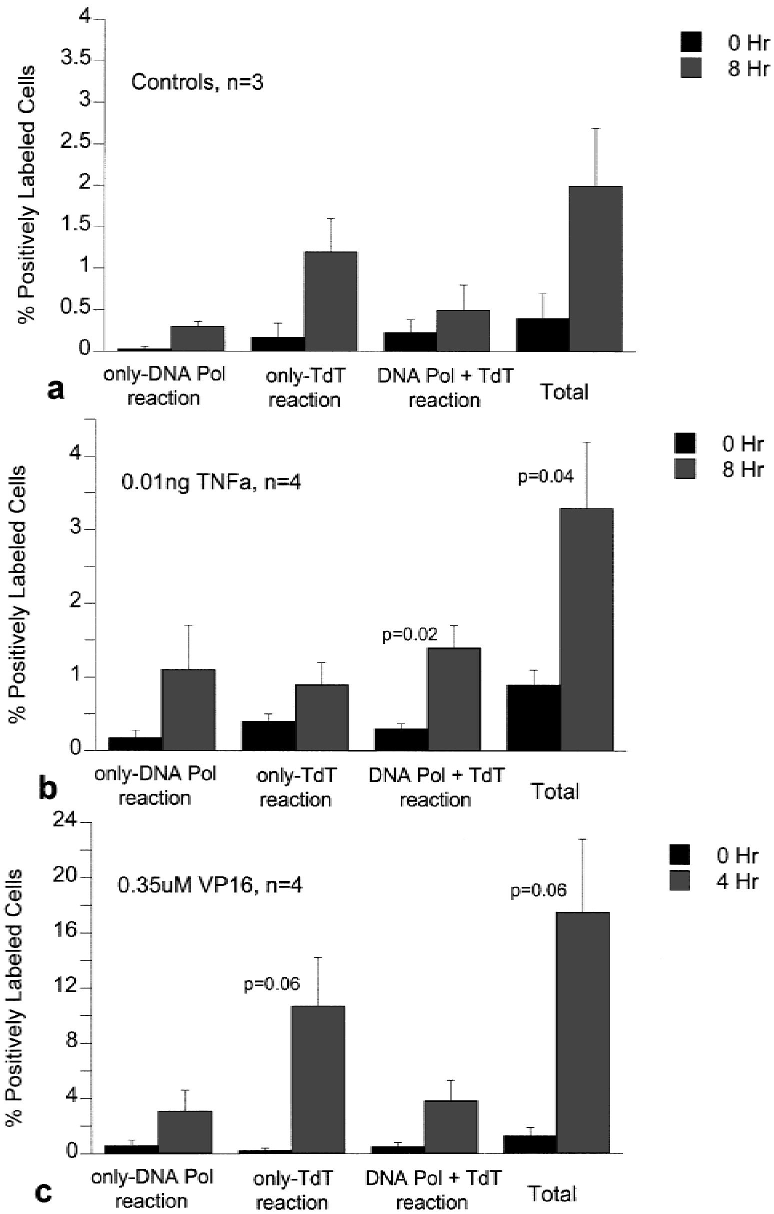


**Fig. 5. Double labeling of HL60 cells: Freshly harvested HL60 cells were treated with 0.01 ng/ml TNF-α (b) or 0.35 μM/l VP16 (c) in RPMI-1640 medium containing 20% FBS. Cells incubated in plain medium served as controls (a). (n = number of experiments) Percent positively labeled cells were determined following DNA Pol/TdT double labeling. Interestingly, untreated cells (a) and VP16-treated (c) cells showed the highest increase in cells labeled with only-TdT reaction (only-blue staining), whereas following TNF-α treatment (b) the highest increase was found in cells double-labeled with DNA Pol + TdT reaction. The net increase in total labeling index was two times higher with TNF-α and almost 10 times higher with VP16 treatment as compared with the untreated cells.**

and others in MDS aspirates [2,4]. Nonetheless, all previous reports by others and us [1–5] are in complete agreement with the fact that MDS show higher apoptosis than normal in either type of specimen. In the present study, the MDS bone marrow cells showed 2–3 times higher propensity than the normal cells to spontaneously undergo apoptosis despite the presence of serum in the medium. When relative percent increase in different labeling patterns of the total net increase was compared in normal, MDS, and HL60 cells, as shown in Figure 6, in normal cells the rate of increase in each individual labeling pattern was comparable. Surprisingly in MDS, the comparative labeling pattern exhibited a similar profile as that demonstrated by the TNF-α–treated HL60 cells. Both showed the highest increase in cells double labeled with DNA Pol and TdT, and the least in those labeled by only-TdT reaction. Our earlier studies have shown excessive apoptosis and higher levels of TNF-α in MDS marrows as compared with normal marrows [3]. Using a simultaneous histochemical double labeling for TNF-α and apoptosis, these studies also revealed high preponderance of TNF-α around cells undergoing apoptosis. The present studies thus provide further circumstantial evidence for the possible association of TNF-α with increased incidence of apoptosis in MDS.

**Apoptotic DNA fragments may have unique length but differently staggered ends.** As mentioned earlier, the majority of apoptotic endonucleases appear to be topologically restricted in their action to the internucleosomal linker region of DNA [6–8], which in general ap-

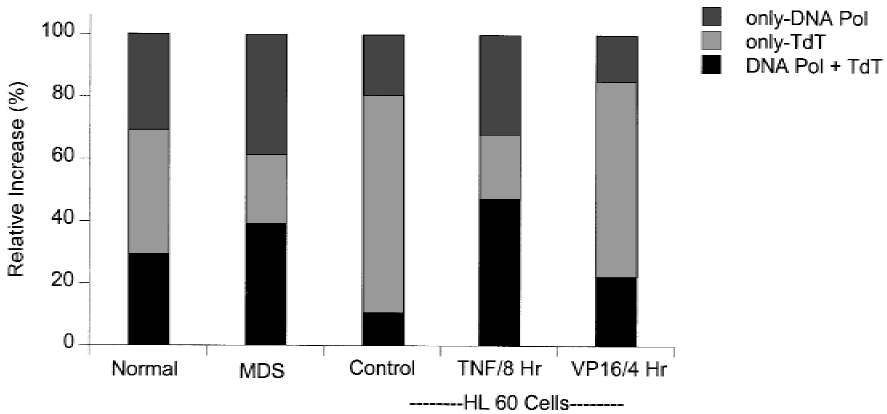


**Fig. 6.** **Comparison of relative percent increase in individual labeling patterns of the total net increase in MDS, normal, and HL60 cells. As illustrated in the figure, whereas normal cells showed comparable increase in the three patterns, MDS cells showed relatively highest increase in cells double labeled with DNA Pol + TdT reaction and least in cells with only-TdT reaction. Interestingly this pattern is matched by TNF-$\alpha$ treatment of HL60 cells. In contrast, untreated and VP16-treated HL60 cells showed the highest increase in cells with only-TdT reaction (only-blue staining).**

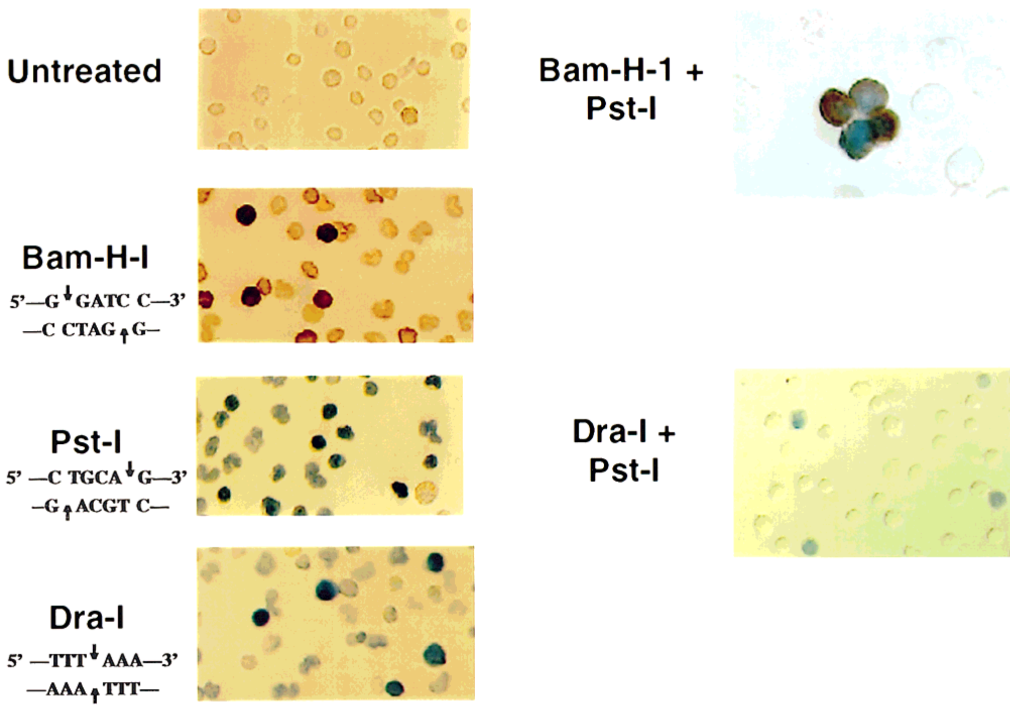


**Fig. 7.** **Double labeling of DNA breaks produced by known endonucleases in normal bone marrow mononuclear cells: The cells were treated with known sequence specific endonucleases—*Bam*HI (causes 3′ recessed breaks with 5′ overhangs—5′-G↓GATCC-3′), *Pst*I (causes 5′ recessed breaks with 3′ overhangs—5′-CTGCA↓G-3′) or *Dra*I (causes blunt ended breaks—5′-TTT↓AAA-3′). (See Methods section for more details). Also, combination treatment with *Pst*I followed by *Bam*HI or the two together, or *Pst*I followed by *Dra*I or together, was performed. As can be seen in this figure, cells treated with *Bam*HI singly showed only brown staining (only-DNA Pol *reaction; original magnification, ×400), while those treated with Pst*I or *Dra*I singly showed only blue staining (only-TdT reaction; original magnification, ×400). Interestingly, cells treated with *Pst*I first followed by *Bam*HI, showed double labeling (DNA Pol + TdT reaction; original magnification, ×1000), whereas a treatment with the two enzymes together showed dominance of *Bam*HI and resulted in only-brown staining (not shown). In contrast, a treatment with *Pst*I + *Dra*I, sequentially or together, showed only blue staining (only-TdT reaction; original magnification, ×400).**

pears to be more sensitive to nucleolytic action than the nucleosomal core [16–18]. As a result, they can potentially give rise to oligonucleosomal length DNA fragments. Does this mean that the action of different apoptotic endonucleases is indistinguishable? Although not directly associated with specific endonucleases, our present investigations for the first time clearly identified differences in the final products of apoptotic endonucleolytic process. Such differences may reflect the unique specificities exerted by the endonucleases in cleaving two strands of DNA. Earlier reports [18–21] support the hypothesis that DNA fragmentation in apoptosis may occur in at least three steps. The first step is the generation of single-stranded breaks that subsequently leads to the

formation of large molecular weight fragments (~50–300 kb) and eventually to small molecular weight fragments (multiples of 180–200 bp). The single-stranded breaks in this case need to occur in close proximity within 14 bases, which then can resemble a double-strand break [18]. Plausibly, therefore, not all endonucleases may cut the two strands of DNA simultaneously although they are committed to double-strand cleavage. Given that the endonuclease nicks one strand at a time, this may give rise to typically staggered ends. In the past DNase I, DNase II, and Staphylococcal nuclease have been shown to cleave DNA around a common dyad axis giving rise to DNA fragments of the size of multiples of 10 nucleotides and having staggered ends [22–24].

Our data on MDS and normal bone marrow aspirate mononuclear cells undergoing spontaneous apoptosis, showed the presence of a unique length of DNA fragments giving rise to a characteristic ladder upon agarose gel electrophoresis. In situ double labeling of these cells with DNA Pol/TdT, however, revealed the differences in DNA fragmentation at a single cell level in these populations. In situ digestion of intact DNA in normal bone marrow and peripheral blood mononuclear cells with known sequence-specific endonucleolytic enzymes assisted in the recognition of differently staggered ends of DNA fragments generated in spontaneous apoptosis of MDS and normal cells, and in induced apoptosis in HL60 cells. Although the action of various nucleases previously has been reported on isolated DNA or in cell-free extracts [22–24], direct cleavage of nuclear DNA in whole cells following treatment with nucleases is being reported for the first time in the present paper. Perhaps the fixation with paraformaldehyde and periodic acid followed by a pretreatment with SSC solution enhanced the permeability of these endonucleases and facilitated their action in whole cells in our experiments. These data suggest that the DNA Pol specific brown labeling in the double labeling of MDS, normal, or HL60 cells could be due to the presence of 3′ recessed DNA fragments, whereas the TdT related blue labeling could be due to the presence of 5′ recessed, and/or blunt-ended DNA fragments. Moreover, the double-labeled pattern could result from the concomitant presence of 3′ recessed DNA fragments with 5′ recessed or blunt-ended DNA fragments.

In a review article on DNA breaks, Eastman and Barry [25] have stated that reparable single-strand breaks occur at high frequency in all cell types in general. Is it possible that the two enzymes used in our double-labeling technique could label these breaks and cause false positivity? It seems unlikely because in our studies the labeling indices observed in spontaneous as well as induced apoptosis were low and a large proportion of nonapoptotic cells, which also could have reparable single-strand breaks, were left unlabeled by either enzyme. Furthermore, although DNA Pol has exonucleolytic activity, it does not appear to result in false positivity either because previous studies by our group (unpublished data) and Wijsman et al. [10] showed that the Klenow fragment of DNA Pol that lacks the exonucleolytic activity labels apoptosis by comparable sensitivity. Furthermore, TdT, which usually requires a single-stranded DNA primer, in the presence of $CO^{2+}$, however, adds nucleotides at the 3′-OH ends of all forms of duplex DNA [26]. Clearly, therefore, the differential labeling of apoptosis by the two enzymes, in most likelihood, may be due to the presence of DNA fragments with differently staggered ends.

**Specific DNA fragmentation patterns are not related to the stage of apoptosis.** Electron microscopy broadly defined three recognizable stages of nuclear changes in apoptosis: 1. The beginning of chromatin condensation and collapse on the inner nuclear membrane termed nuclear margination as the very early stage; 2. Subsequent extensive condensation and formation of larger clumps located in the center of the nucleus as the intermediate stage; and 3. Complete karyorrhexis and apoptotic body formation as the end stage of apoptosis [27]. The in situ DNA end-labeling techniques described here offer the advantage of visualizing all these stages under light microscopy. Interestingly, in our double-labeling experiments each labeling pattern was found at all the stages of apoptosis described above (Fig. 2). This indicates the maintenance of a specific DNA fragmentation pattern throughout the process of apoptosis. In the past, demonstration of initial formation of large high molecular weight DNA fragments subsequently getting cleaved into small low molecular weight fragments, raised the possibility of a sequential participation of multiple endonucleases in the process of apoptotic DNA degradation [20]. Maintenance of only-DNA Pol–specific brown labeling or only-TdT–specific blue labeling patterns at all stages of apoptosis in the present investigations, suggests that either a single endonucleolytic activity or multiple activities with similar specificity for DNA cleavage may participate in DNA degradation. The results of Barry et al. [19] support this view, which showed the formation of high molecular weight DNA fragments and their conversion into low molecular weight fragments at a lowered pH in HL60 cells undergoing apoptosis where only acidic endonuclease (like DNase II) could possibly participate. Interestingly, in the past, DNase II has been shown to generate both types of fragment [28].

The double-labeling pattern, however, clearly demonstrates participation of at least two endonucleolytic activities, albeit with distinct specificities. Again, since this labeling pattern was found at all stages of apoptosis as well, it appears that the two activities may not be stage specific. In experiments in which cells were treated with *Bam*HI (generating 3′ recessed DNA fragments) and *Pst*I (generating 5′ recessed DNA fragments), the double labeling could be observed only when cells were incubated

sequentially with *Pst*I first. A simultaneous treatment with the two enzymes showed dominance of *Bam*HI giving only brown labeling. Further investigation is needed to determine if multiple endogenous endonucleolytic activities in MDS, normal, and HL60 double-labeled cells also operate in a specific sequence.

Hence, distinct DNA fragmentation patterns may not be specific to the stage of apoptotic death but may rather depend on an inherent property of the cell. The presence of three DNA fragmentation patterns in apparently homogeneous population of HL60 cells may suggest the emergence of biochemical variants on long-term in vitro maintenance. On the other hand, the bone marrow mononuclear cells in MDS and normal subjects represent a highly heterogeneous population of different phenotypes as well as a repertoire of maturation stages in each case. The DNA fragmentation pattern may thus be specific to a lineage or maturation stage. Anzai et al. [29] have shown that $Mg^{++}$-dependent endonuclease is expressed during apoptotic death of hematopoietic progenitor cells. It is lost upon differentiation, and the mature granulocytes undergoing apoptosis show $Ca^{++}$-$Mg^{++}$–dependent and acidic endonucleases. Furthermore, circulating mature neutrophils show only acidic endonuclease in their apoptosis [30]. Anzai and associates [29] have also shown a shift of $Ca^{++}$-$Mg^{++}$–dependent endonuclease activity to $Mg^{++}$-dependent activity correlating with a rise in immature cell count in MDS [31] and the exclusive presence of $Mg^{++}$-dependent activity in myeloid leukemia where immature myeloid blasts constitute the majority of bone marrow cell population [32].

**Different DNA fragmentation patterns could preferentially be induced by specific effectors of apoptosis.** The two inducers of apoptosis, TNF-α and VP16, used in the present investigation showed specific preferences for DNA fragmentation patterns. Whereas VP16 promoted apoptosis involving 5′ recessed and/or blunt-ended fragments labeled by only-TdT reaction, TNF-α on the other hand, showed enhancement of apoptosis involving a copresence of 3′ recessed fragments with 5′ recessed and/or blunt ended fragments double labeled by DNA Pol and TdT reactions. Induction of differential DNA fragmentation patterns in the same cell line by the two agents studied may indicate activation of distinct endonucleases, perhaps in unique biochemical pathways. An earlier report by Tanuma and Shiokawa [33] reinforces this view by showing that gamma irradiation of rat thymocytes resulted in selective activation of $Ca^{++}$-$Mg^{++}$–dependent endonuclease and in fact reduction of acidic endonuclease(s) present concomitantly in these thymocytes.

The demonstration of distinct DNA fragmentation patterns in apoptosis may reflect the existence of multiple apoptotic pathways. Recent reports give an impression that most triggers of apoptosis converge on a final protease pathway [34,35]. It would be interesting to examine how this final common pathway activates distinct endonucleases and their respective DNA fragmentation patterns.

The present studies provide new insights into the biology of DNA fragmentation in apoptosis, which may have significant clinical implications. A particular strength of the current study is the ability to examine these patterns in situ thereby allowing for simultaneous lineage recognition in tissues such as the bone marrow which contains a variety of hematopoietic cells. If DNA fragmentation is tissue/cell specific and if different chemotherapeutic agents show preferential induction of endonucleases with their respective DNA fragmentation patterns, then screening of tissues and agents for the expression and/or induction of different DNA fragmentation patterns would provide relevant information for designing more specific therapeutic regimens in cancer treatment. The demonstration of TNF-α–inducible DNA fragmentation pattern in MDS may indicate that perhaps anti–TNF-α (or anticytokine) therapy could be of special benefit to these patients. Indeed, our recent clinical studies in MDS patients demonstrating a favorable clinical response to an anticytokine regimen comprised of pentoxifylline, ciprofloxacin, dexamethasone, and amifostine are compatible with our present biologic observations in the BMs of these patients [36].

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